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Cloning and Expression of the Rat Vacuole Membrane Protein 1 (VMP1), a New Gene Activated in Pancreas with Acute Pancreatitis, Which Promotes Vacuole Formation

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To characterize the emergency program set up by pancreatic cells in response to pancreatitis, we established the phenotype of the pancreatitis-affected pancreas by characterizing a large number of its transcripts. In this report, we describe the cloning, sequencing, and expression pattern of a new gene, named VMP1 (vacuole membrane protein 1). The VMP1 mRNA codes for a putative protein of 406 amino acids. *In situ* hybridization studies revealed that pancreatic expression of VMP1 mRNAs was restricted to the acinar cells. Interestingly, VMP1 mRNA was also overexpressed in kidney after transient ischemic injury. However, many healthy tissues express VMP1 mRNA. Structure analysis suggested that VMP1 is a transmembrane protein with six hydrophobic regions. VMP1/EGFP fusion protein was located to the Golgi apparatus and the endoplasmic reticulum area. Expression of this protein promoted the formation of intracytoplasmatic vacuoles and VMP1/EGFP was located to the membranes of these vacuoles. Cells overexpressing this protein died after 48 h. In conclusion, we have identified a new stress-induced gene which codes for a transmembrane protein that, when overexpressed, promotes formation of intracellular vacuoles followed by cell death. © 2002 Elsevier Science

Acute pancreatitis is the most frequent disease of the pancreas. The spectrum of acute pancreatitis can range from mild edematous to severe necrotizing (1). One of the most interesting hypotheses for the pathogeny of the disease is that autodigestion of the gland occurs when hydrolytic enzymes are unduly activated within the pancreas rather than into the intestinal lumen (2, 3). Living organisms respond at the cellular level to stress or pathological aggression by altering the normal pattern of protein synthesis (4–8). That change is characterized by a dramatic induction of stress proteins with concomitant inhibition of the normal array of cellular proteins. Studies in animals and humans, performed during the acute phase of pancreatitis, demonstrated that the content and secretion of pancreatic enzymes, which are potentially harmful, were generally reduced, as part of a defense mechanism. Conversely, other genes were strongly activated during the acute phase of the disease (9, 10). Therefore, like other organs, the pancreas exposed to acute stress seems to trigger a stringent emergency program that helps the gland fight the aggression and, consequently, protects the organism from the deleterious effects of pancreatitis.

The aim of our research is to characterize at the molecular level the pancreatic emergency program set up in response to pancreatitis. We developed a strategy in which the phenotype of the pancreas with acute pancreatitis was established by characterization of a large number of its transcripts. Such a cDNA collection represents a reservoir from which transcripts involved in the emergency response can be identified on the basis of their expression patterns. In this report, we characterized a novel membrane protein, named VMP1, that is strongly and rapidly induced in pancreas during acute pancreatitis. This new protein is located at the Golgi apparatus and the endoplasmic reticulum area of acinar cells. Its overexpression promotes formation of intracellular vacuoles and cell death.

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MATERIALS AND METHODS

Pancreatic cDNA library, sequencing, and database analysis. Construction and analysis of the EST library of pancreas with pancreatitis was previously reported (11). The cDNA inserts of 1536 phagemids were partially sequenced by Genome Express (Grenoble, France). Sequences from the EST library were compared with each other to determine their frequency of appearance. Each sequence was also compared with all sequences available in GenBank using the BLAST network service. Only the 10 best reports, sorted by statistical significance using Poisson P value were taken into account. The rat VMP1 cDNA insert of clone 10F5 was completely sequenced by Genome Express (Grenoble, France).

Rat VMP1 mRNA expression. Male Sprague–Dawley rats were used in all experiments. Animals were housed with free access to food and water. Experiments were performed according to the standard ethical and legal guidelines and with the permission of the local committee for the inspection of animal experiments. Acute pancreatitis was induced in rats weighing 200–250 g. Edematous pancreatitis was induced by two intraperitoneal injections of cerulein (40 μg/kg) given at a 30-min interval. The animals were killed 1, 3, 6, 9, 12, 15 and 18 h after the first injection. Pancreas, intestine, liver, spleen, lung, kidney, stomach, thymus, brain, testis, thyroid, ovary, placenta and retina were processed according to Chirgwin *et al.* (12) for RNA preparation. Renal ischemia was performed according to the protocol of Schuraer *et al.* (13). Briefly, the right kidney was approached through a flank incision and the renal artery was temporarily blocked with a vascular clamp for 30 min. Fifteen hours later, the rats were sacrificed and the right (ischemic) and left (control) kidneys were removed and immediately processed for total RNA isolation. RNA preparation for ontogeny was performed as follows: 19- and 21-day old rat fetuses, newborns, and 3-, 5-, 7-, 9-, 13-, 15-, 19-, 21-, 23-, 25-, 35-, 45-, and 90-day-old rats were used. Fetal pancreas were obtained from timed pregnant rats 19 and 21 days after appearance of vaginal plug, and pancreas from several animals (both male and female) were pooled. Rats were sacrificed by decapitation, and their pancreas were rapidly removed and stored into liquid nitrogen until processing for total RNA isolation.

Northern blots. Twenty micrograms of RNA was submitted to electrophoresis on a 1% agarose gel and the gel eventually vacuum blotted onto a Hybond N membrane (Amersham–Pharmacia Biotech). The filters were hybridized with the corresponding ^{32}P-labeled probes for 16 h at 65°C in 5× SSPE (1× SSPE is 180 mM NaCl, 1 mM EDTA, 10 mM NaH_2PO_4, pH 7.5), 5× Denhardt's solution, 0.5% SDS and 100 μg/ml single-stranded herring sperm DNA. Then, the filters were washed four times for 5 min at room temperature in 2× SSC (1× SSC is 150 mM NaCl, 15 mM sodium citrate), 0.2% SDS twice for 15 min at 50°C in 0.2× SSC, 0.2% SDS and once for 30 min in 0.1× SSC at 50°C.

In situ hybridization analysis for expression of rat VMP1 mRNA. Preparation of probes: The rat VMP1 complementary RNA (cRNA) probe was transcribed from the 1858-bp *NotI–EcoRI* rat VMP1 cDNA fragment (clone 10F5), inserted into plasmid pT7T3D (Amersham–Pharmacia Biotech). The templates were linearized with *EcoRI* and T3 RNA polymerase was used for *in vitro* antisense transcription, or NotI and T7 RNA polymerase for the sense cRNA transcription. After linearization, cRNA probes were labeled with DIG UTP (Roche Molecular Biochemicals). The *in situ* hybridization method was as described by Kuramitsu *et al.* (14) with modifications described in Mallo *et al.* (11). As a negative control, the sections were treated with RNase A prior to prehybridization, and a second negative control was performed using the rat VMP1 sense RNA as probe. After hybridization, unbound probe was digested with RNase A (20 μg/ml) for 30 min at 37°C. The post-hybridization washes were all performed at 37°C, and consisted of two washes in 2× SSC, two in 0.2× SSC, and one in 0.1× SSC (each for 15 min). The hybridization signal was detected according to the instructions of the Dig nucleic acid detection kit (Roche Molecular Biochemicals).

Cell culture and transfection of the rat VMP1 cDNA. AR4-2J pancreatic acinar cells were used after 48 to 55 passages. The cells were routinely cultivated at 37°C in a 5% CO_2, 95% air atmosphere in Dulbecco's modified Eagle medium containing 10% (v/v) fetal calf serum (Life Technologies, Inc.), 4 mM L-glutamine, 50 units/ml penicillin and 50 μg/ml streptomycin. Cos7, HeLa, NIH 3T3, and IEC6 cells were maintained under the same culture conditions as AR4-2J cells except that insulin (10 μg/ml) was added to IEC6 cells. When cells reached 80–90% confluence, they were dissociated with 0.05% trypsin and 0.02% EDTA in Puck's saline A and replated into 100-mm petri dishes. The full-length rat VMP1 cDNA was subcloned, either into the *EcoRI–BamH*I restriction sites of the mammalian expression vector pEGFP-N1 (Clontech) to generate a fusion protein with the enhanced green fluorescent protein (EGFP), or into the *EcoRI–BamH*I restriction sites of the mammalian expression vector pcDNA4 (Invitrogen) to generate a fusion protein with the V5 epitope. In both cases the constructs are located downstream the cytomegalovirus promoter. pEGFP-N1 confers resistance to geneticin (G-418 sulfate) and pcDNA4 to Zeocin. The recombinant plasmids were named pVMP1/EGFP or pVMP1/V5. As control, we used the pEGFP-N1 or pcDNA4 empty vectors. The plasmids were transfected into Cos7, HeLa, IEC6, NIH 3T3 and AR4-2J cells with Fugene, following instructions of the manufacturer (Roche Molecular Biochemicals).

Membrane isolation and Western blot. Membrane proteins were prepared as follows: cultured cells were washed and homogenized with a motor driven Teflon pestle homogenizer in ice cold SBE buffer (250 mM sucrose, 1 mM EGTA, 10 mM Hepes/KOH (pH 7.5) containing aprotinin (60 ng/ml), benzamidine (1 mM), leupeptine (4 mg/ml), phenylmethylsulfonyl fluoride (0.8 mM) and soybean trypsin inhibitor (20 mg/ml). The homogenates were centrifuged twice at 500*g* for 15 min. The resulting supernatant was centrifuged at 100,000*g* for 1 h or treated with Nonidet P-40, 0.2 M Na_2CO_3 pH 11.0 or 1.5 M NaCl prior to centrifugation. The membrane pellet was resuspended in ice-cold SMB buffer, aliquoted and stored at −80°C. The supernatant was stored in the same conditions. All steps were performed at 4°C. Protein concentration was measured by the Bradford method. Each sample (60 μg) was submitted to SDS–polyacrylamide gel electrophoresis (10% acrylamide, 0.5% bisacrylamide) and transferred to a nitrocellulose membrane. The first antibody was against the V5 epitope (Invitrogen) used at a 1:4000 dilution and the second antibody was a peroxidase-labeled anti-rabbit IgG antibody provided with the ECL kit (Amersham–Pharmacia Biotech). Immunoblotting was performed using the ECL kit and membranes were exposed to a Kodak Biomax film for about 1 min.

Cell viability upon VMP1 expression (colony formation). Cos7 cells (10^6) were plated into 30-mm petri dishes and transfected 24 h later with the VMP1/V5 plasmid (3 μg) or with the pcDNA4 empty vector as control using Fugene as described above. The transfected cells were selected in Zeocin (0.3 mg/ml) for 10 days and stained with crystal violet to assess the number of colonies. That experiment was repeated three times.

FIG. 1. Nucleotide sequence and predicted amino acid sequence of VMP1. (A) The predicted amino acid sequence of VMP1 is shown above the nucleotide sequence. Amino acid and nucleotide residues are numbered to the right of each sequence. In the nucleotide sequence, the putative N glycosylation site is indicated by ♦, protein kinase C phosphorylation sites are indicated by ♣, and casein kinase II phosphorylation sites are indicated by ♥. (B) Kyte and Doolittle window showing the six putative transmembrane helices.

A

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GAATTCCGGGCTTCCAGGATCCTGCGATCCGGAGAAGCCGGGGTCTGAGCGGCTCCTCCAGAGTTACTGAT    71

                    M  A  E  N  Q  T  N  C  D  Q  R  R  G  A  M  S    16
CTATGAA ATG GCA GAG AAT CGA ACA AAT TGT GAC CAG AGA CGT GGA GCA ATG AGT    126

 K  E  Q  H  N  G  S  F  T  D  F  S  S  V  N  E  K  K    34
AAG GAA CAG CAT AAT GGG AGT TTC ACA GAC CCC TCT TCA GTA AAT GAG AAG AAG    180

 R  R  D  R  E  E  R  Q  N  I  V  L  M  R  Q  P  L  I    52
AGA AGG GAT CGG GAA GAA AGA CAG AAT ATC GTC CTG TGG AGA CAG CCA CTC ATT    234

 T  L  Q  Y  F  S  L  S  T  L  V  V  L  K  E  M  T  S    70
ACC TTG CAG TAT TTC TCT CTG GAA ACT CTT GTA GTT TTG AAG GAA TGG ACC TCA    288

 K  L  W  R  R  Q  S  W  V  V  S  F  F  L  L  L  A  A    88
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 L  V  A  F  Y  Y  V  R  G  A  H  Q  Q  Y  V  Q  R  I    106
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 Y  L  E  A  Q  R  Q  K  L  H  K  R  S  K  A  G  T  A    358
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 Q  G  E  M  W  L  S  W  T  F  E  K  L  V  V  A  M  V    376
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 C  Y  F  I  L  S  I  I  M  S  M  R  Q  S  Y  A  K  R    394
TGT TAC TTT ATC CTG TCT ATC ATT AAC TCC ATG CGA CAA AGC TAT GCC AAA CGA   1260

 I  Q  Q  R  L  N  S  R  R  K  T  K  *                  407
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CTG CAG AAG TTA GCG TGG TGG AGC CTG CCT TAC ATC GGG AGG GCT CTC ACC AGG   1368

AAG GAA GAT CCC CAT TTC CAA GCT GTA CTG ATT TTT AAA ACA TTT CTC CCT GAA   1422

AGC AGT TTA GTC CAC ATT TTA CAC TGA CAT ACT TTT GCT TTC TTT GTT AAG GTA   1476

AGG TCT CCA CCC TCG ATT CAA TCC ACA TTG TAT TCC TTA GGG TGG ATA TGA TGT   1530

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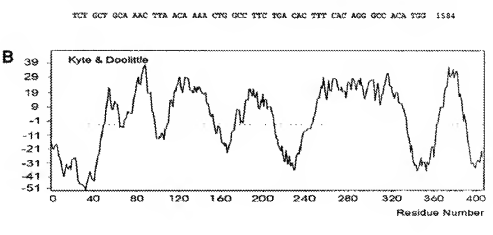
B



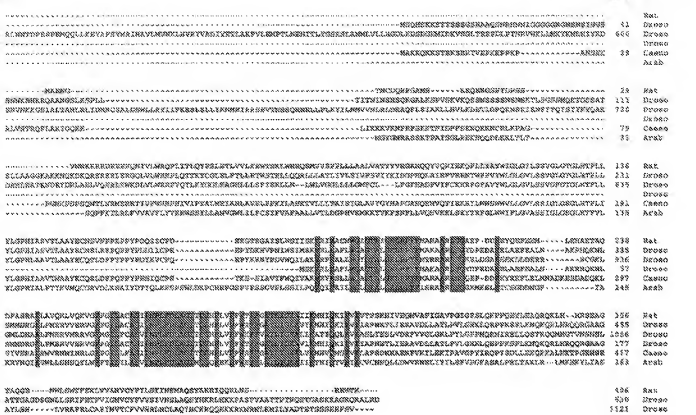


FIG. 2. Sequence comparison between rat VMP1 and its homologues in other species. Alignment of rat VMP1 (ratVMP) and the homologues from *Arabidopsis thaliana* (Arab), *Caenorhabditis elegans* (Caenu) and *Drosophila* (Drosp). The boxed sequences represent residues conserved between rat VMP1 and its homologues.

RESULTS

Cloning the Rat VMP1 mRNA

A cDNA library of 4.1×10^5 clones was constructed from the polyadenylated RNA fraction purified from a rat pancreas with acute pancreatitis. From this cDNA library, 1536 randomly selected clones were partially sequenced and the resulting sequences were compared with the GenBank database. Among these clones, 256 could not be related to any sequence in the database. Expression of these mRNAs during acute pancreatitis was systematically analyzed by Northern blot. Expression analysis of clone 10F5 showed an interesting pattern. This mRNA is strongly and rapidly activated after the induction of experimental acute pancreatitis. Sequencing of the cDNA insert of clone 10F5 was completed. The insert was 1858 bp long. A single open reading frame was found in the corresponding mRNA sequence (Fig. 1A). Three methionines at positions 79, 121, and 310 were potential initiation sites for translation. In vertebrates, however, protein synthesis typically starts from the upstream methionine (15). Hence, synthesis of VMP1 very probably starts at nucleotide 79. The 3′ region consisted of a TAA stop codon at position 1297 followed by 562 nucleotides of untranslated message [excluding the poly(A) stretch]. No polyadenylation recognition site (AATAAA) was found. The VMP1 mRNA codes for a protein of 406 amino acids, with a theoretical *p*I of 6.28. The predicted molecular weight is 45,901 Da.

SOSUIsignal (http://sosui.proteome.bio.tuat.ac.jp/sosuisignal/sosuisignal_submit.html) analysis of the deduced VMP1 primary structure revealed that it has no signal peptide. However, presence of 6 transmembrane helices (Fig. 1B) are typical of a transmembrane protein. The amino acid sequence was compared to the GenBank database and was aligned with other transmembrane proteins from *Drosophila* (Accession Nos. AAF46618, AAF50018, and AAF46619), *Caenorhabditis elegans* (Accession No. T26642) and *Arabidopsis thaliana* (Accession No. AAB71442) (Fig. 2).

VMP1 mRNA Expression during the Course of Acute Pancreatitis

Pancreatic RNA was obtained from rats at different times after induction by cerulein of experimental acute pancreatitis. As shown in Fig. 3, Northern blot analysis

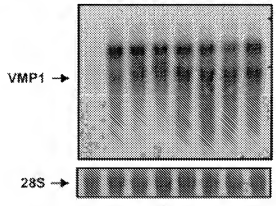


FIG. 3. VMP1 expression in pancreas with experimental acute pancreatitis. Acute pancreatitis was induced in rats weighing 200–250 g by two intraperitoneal injections of cerulein. Animals were killed at the indicated times following the second injection and their pancreas was processed for RNA preparation. Identical amounts of total RNA were blotted after electrophoresis on agarose-formaldehyde gels. The filter was probed with ^{32}P-labeled VMP1 cDNA (top). As control, the same filter was washed and hybridized with ^{32}P-labeled 28S ribosomal RNA (bottom).

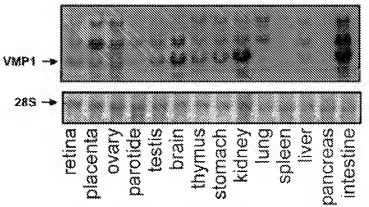


FIG. 5. Tissue expression of VMP1 mRNA. RNA samples subjected to analysis were prepared from the following tissues: retina, placenta, ovary, parotid, testis, brain, thymus, stomach, kidney, lung, spleen, liver, pancreas and intestine. For each tissue, 20 μg of total RNA was blotted after electrophoresis on agarose-formaldehyde gels. The filter was probed with ^{32}P-labeled VMP1 cDNA (top). As control, the same filter was washed and hybridized with ^{32}P-labeled 28S ribosomal RNA (bottom).

with the VMP1 cDNA probe revealed a very low level of expression at time 0. Significant induction was observed after 30 min. It was maximal after 1 h and remained activated during the whole study (18 h). It is noteworthy that the *VMP1* gene generates two major transcripts (1.9 and 2.7 kb), both being strongly induced in the inflamed pancreas.

VMP1 mRNA Expression in the Developing Rat Pancreas

As shown in Fig. 4, high VMP1 mRNA levels were observed in the fetal pancreas on day 19 and remained elevated in the newborn until day 11. Then, it suddenly decreased and remained low until day 23, after which it was no longer detectable.

Extrapancreatic VMP1 mRNA Expression

Total RNA extracted from intestine, liver, spleen, lung, kidney, stomach, thymus, brain, testis, thyroid, ovary, placenta, and retina obtained from control rats (Fig. 5) or after 24 or 48 h of pancreatitis were probed with the ^{32}P-labeled VMP1 cDNA. Both VMP1 transcripts (1.9 and 2.7 kb) were highly expressed in control intestine, kidney, ovary and placenta, moderately expressed in liver, lung, stomach, thymus, brain, and testis and, slightly expressed in control thyroid and retina. A third band of about 3.5 kb was observed in intestine, liver, lung, kidney, stomach, thymus, ovary and placenta. Expression in other tissues than pancreas was not affected in animals with acute pancreatitis (data not shown).

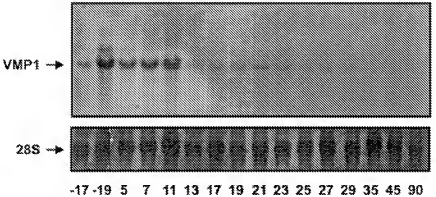


FIG. 4. Developmental expression of VMP1 mRNA in pancreas. Twenty micrograms of total RNA from pancreas of 19- and 21 day-old fetus (F17 and F19), from newborn pancreas (NB), and from pancreas of rats aged 5, 7, 11, 13, 17, 19, 21, 23, 25, 27, 29, 35, 45, and 90 days was blotted after electrophoresis on agarose-formaldehyde gels. The filter was probed with ^{32}P-labeled VMP1 cDNA (top). As control, the same filter was washed and hybridized with ^{32}P-labeled 28S ribosomal RNA (bottom).

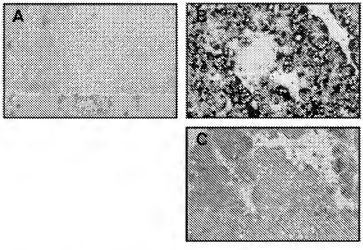


FIG. 6. *In situ* hybridization of VMP1 mRNA in pancreas with cerulein-induced pancreatitis. Acute pancreatitis was induced in rats by cerulein. Animals were killed and the pancreas was removed and immediately fixed in formalin. Five μm thick, paraffin embedded tissue sections were deparaffinized, hydrated, permeabilized and prehybridized as described under Materials and Methods. Hybridization was performed overnight in the presence of 5–10 ng of the VMP1 DIG-UTP cRNA probe. Sections were washed and investigated with the Dig nucleic acid detection kit (Roche Molecular Biochemicals). (A) Healthy pancreas shows not signal. (B) This micrograph illustrates the VMP1 mRNA hybridization signal observed in the cytoplasm around the nucleus of acinar cells in pancreas after 6 h cerulein induce pancreatitis. No expression was detected in islet cells. (C) Control hybridization of experiment shown in B, with digoxigenin labeled sense cRNA probe. No signal was detected. Original magnification, ×40.

In Situ Hybridization of VMP1 mRNA in the Rat Pancreas with Acute Pancreatitis

In situ hybridization was performed to identify which cell type expressed the VMP1 transcript in pancreas. Pancreatic tissues from control and cerulein-treated rats were hybridized with the digoxigenin-labeled antisense VMP1 RNA. A strong labeling was observed in the acinar cells (Fig. 6A). By contrast, VMP1 remained undetectable in the islets of Langerhans, ducts, inflammatory infiltrate, and stromal tissue in pancreas. Control hybridization with a digoxigenin-labeled sense RNA probe (Fig. 6B) or with an antisense RNA on RNase A-treated tissue sections showed no specific hybridization. Pancreas from control rats showed no signal.

Induction of VMP1 mRNA Expression in Kidney

To look whether VMP1 induction was specific to the injured pancreas, we monitored its expression in the postischemic kidney. In our experimental model, the left kidney of the rats was submitted to a 30 min ischemia, the right kidney being used as control, and expression of VMP1 mRNA was studied 16 h later. Results are shown in Fig. 7. VMP1 mRNA expression is induced by ischemic treatment. VMP1 mRNA expression was low in the right (control) kidney, but strong in the left (ischemic) kidney, in which both transcripts (1.9 and 2.7 kb) were overexpressed. Pancreas of the same animals showed no VMP1 mRNA induction.

VMP1 Is a Transmembrane Protein

Cos7 cells allowing expression of V5-tagged VMP1 protein were used for subcellular fractionation and Western blot experiments. Figure 8 shows results of Western blot analysis of V5-tagged VMP1. Protein was detected in membrane preparations, the signal persisting after NaCl-treatment or exposure to pH 11.0.

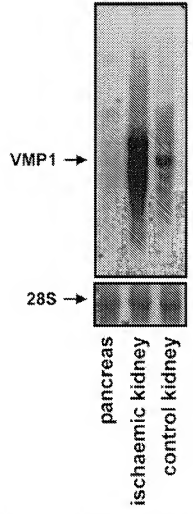


FIG. 7. VMP1 mRNA overexpression in kidney after ischemia. To induce renal ischemia, the right kidney was approached through a flank incision and the renal artery was blocked with a vascular clamp for 30 min, after which the clamp was removed. Fifteen hours later, the rats were sacrificed and the right (ischemic) and the left (control) kidney and the pancreas were removed and immediately processed for total RNA isolation. Twenty μg of total RNA was blotted after electrophoresis on agarose-formaldehyde gels. The filter was probed with ^{32}P-labeled VMP1 cDNA (top). As control, the same filter was washed and hybridized with ^{32}P-labeled 28S ribosomal RNA (bottom).

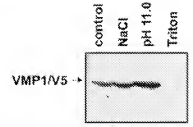


FIG. 8. Western blot of VMP1/V5 in cellular membranes. Cultured cells were washed and homogenized in ice-cold SBE buffer as described under Materials and Methods. The homogenates were centrifuged and the resulting supernatant was centrifuged at 100,000*g* for 1 h or treated with Nonidet P-40, 0.2 M Na_2CO_3, pH 11.0, or 1.5 M NaCl prior to centrifugation. The membrane pellet was resuspended in ice-cold SMB buffer, aliquoted and stored at −80°C. The supernatant fraction was stored similarly. Each sample (60 μg) was submitted to SDS–polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The antibody against the V5 epitope was used at 1:4000. Immunoblotting was performed using the ECL kit.

VMP1 Overexpression Induces Vacuole Formation and Cell Death

The intracellular localization of VMP1 was assessed by transfecting Cos7 cells with a vector allowing expression of an EGFP-tagged VMP1 protein and direct monitoring of EGFP fluorescence. Figure 9 shows that VMP1 is located in the Golgi apparatus and the endoplasmic reticulum area, and that its expression induces formation of vacuoles. VMP1 is integrated into the membranes of these vacuoles. This is why we named the protein Vacuole Membrane Protein 1. The same fusion plasmid was transfected into other cell lines with similar results (data not shown). However, after 48 h of VMP1 overexpression, transfected cells died progressively, with morphological evidence of apoptosis (Fig. 9). To confirm the lethal effect of VMP1 expression, we used Cos7 cells allowing expression of the VMP1 V5-tagged protein and monitored the effects of VMP1 expression in colony formation assays. As a control, Cos7 cells transfected with the pcDNA4 empty vector were used. As shown in Fig. 10, cells transfected with the empty vector formed abundant colonies. On the contrary, the number of colonies formed by cells transfected with the VMP1/V5 plasmid was dramatically reduced.

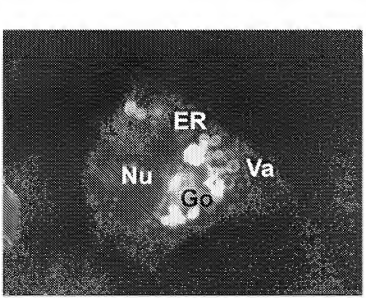


FIG. 9. Subcellular localization of the VMP1/EGFP fusion protein in Cos7 cells. Cos7 cells were transfected with the plasmid pVMP1/EGFP using the Fugene reagent. VMP1 expression was revealed by direct green fluorescence of the VMP1/EGFP fusion protein. Fluorescence microscopy images show that VMP1/EGFP expression induced cytoplasmic vacuole formation. VMP1/EGFP is expressed in the endoplasmic reticulum and Golgi apparatus area and integrated in the vacuole membrane.

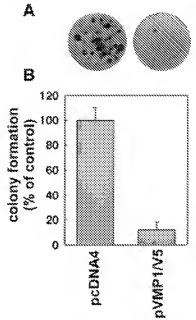


FIG. 10. Colony formation assay. The full-length rat VMP1 cDNA was subcloned into the pcDNA4 vector to generate a fusion protein with the V5 epitope. Cos7 cells (10^6) were plated into 30-mm petri dishes and transfected 24 h later with the pVMP1/V5 plasmid or with the pcDNA4 empty vector as control, using Fugene reagent. The transfected cells were selected in Zeocin (0.3 mg/ml) for 10 days, stained with crystal violet (A), and the number of colonies after that period was quantified (B). Vertical bars show the average numbers of colonies (±SD) for three independent transfections, expressed as percentages of control (pcDNA4).

DISCUSSION

We have previously reported that gene expression is strongly altered in pancreas during the acute phase of pancreatitis (10, 16). These phenotypic changes could enable the pancreas to protect itself against the acute attack of pancreatitis or, conversely, participate in the pathophysiological mechanism of the disease. Therefore, identifying the genes involved in cell response to pancreatitis could lead to new strategies in the treat-

ment of the disease. Using a systematic approach, we identified by sequencing its cDNA a new protein that we named VMP1. It was strongly expressed in the pancreas of rats suffering from acute pancreatitis. Our studies indicated that VMP1 is a transmembrane protein located at the Golgi apparatus and endoplasmic reticulum area and that its overexpression induces vacuole formation and cell death.

In vivo studies demonstrated that VMP1 is an acute phase protein activated in cells submitted to aggression. VMP1 mRNA was strongly overexpressed early after induction of pancreatitis and remained elevated during the whole acute phase. We found similar increases of VMP1 mRNA upon induction of severe acute pancreatitis by retrograde taurocholate injection and induction of mild pancreatitis upon cerulein infusion as well, suggesting that intensity of the disease does not influence *VMP1* gene expression. Interestingly, the rat *VMP1* gene generated in pancreas two major transcripts of 1.9 and 2.7 kb, which might be the result of an alternative splicing. Both transcripts were strongly induced in the inflamed pancreas, suggesting that the splicing mechanism was not regulated by cellular injury. Also, *in situ* hybridization assays demonstrated that VMP1 mRNA expression during acute pancreatitis is exclusively activated in pancreatic acinar cell, indicating that *VMP1* gene activation is part of the acinar cell response to aggression. Although VMP1 mRNA was hardly detectable in the normal pancreas from adult rats, VMP1 mRNA expression, like other acute phase proteins, was evidenced in pancreas during development. Moreover, *VMP1* gene induction in response to aggression was not limited to the pancreas with acute pancreatitis. In fact, kidney submitted to ischemia also overexpressed the *VMP1* gene. Yet, we also noticed that VMP1 mRNA expression is not restricted to injured tissue because we found it constitutively expressed in some healthy tissues. This fact is reminiscent to other pancreatitis-induced genes such as PAP (17), clusterin (18), p8 (11), PIP49 (19), PC3/TIS21/BTG2 (20) previously described by us.

Analysis of the deduced VMP1 primary structure showed that VMP1, which has no signal peptide, is probably a membrane protein with 6 putative transmembrane helices. When homology protein searches were performed against databases, conceptual translation products in Arabidopsis thaliana, *Caenorhabditis elegans* and *Drosophila* were found with high identity scores. The sequence conservation between these distantly related species suggests that VMP1 function is important.

The amino acid sequence predicts that VMP1 may be located in the membrane of the endoplasmic reticulum. There is no a ER-retention motif (KDEL) (21) but a KKXX-like ER-membrane retention signal (EEKT) motif is present at the C terminus of VMP1, resulting in a K-NN prediction of 55.6% for endoplasmic reticulum localization. Experimental data confirmed this prediction. Cell fractionation experiments indicated that VMP1/V5 was expressed in the membrane fraction. Moreover, VMP1/V5 remained in membrane preparation after NaCl or pH 11.0 treatment indicating that VMP1 is a transmembrane protein. Furthermore, localization experiments using EGFP tagged VMP1 in several cell lines showed that VMP1 is expressed at the endoplasmic reticulum and Golgi apparatus areas.

It is noteworthy that *in vitro* experiments using EGFP-tagged VMP1 showed that VMP1 promoted formation of intracellular vacuoles and VMP1 was in fact integrated into the membrane of these vacuoles. These observations may be relevant to the pathophysiology of acute pancreatitis. Intracellular vacuole formation has been reported as an early morphological event in acinar cells during acute pancreatitis (22, 23). The mechanism by which vacuoles appear remains unclear. Crinophagy and defective sorting have been tentatively implicated in the genesis of the vacuoles seen after pancreatitis induction (24). Recently, Raraty *et al.* (25) proposed that intracellular vacuole formation is the result of intracellular trypsinogen activation leading to the replacement of zymogen granules by vacuoles. Our results suggest that an alternative mechanism of vacuole formation involving VMP1 expression may occur in acinar cells during the early stage of acute pancreatitis.

After intracellular vacuole formation, cells expressing VMP1 displayed morphological features of apoptosis and died. We confirmed this observation by measuring the colony formation capacity of Cos7 VMP1/V5-transfected cells. As expected, cells expressing VMP1 were unable to develop colonies after appropriate antibiotic selection, which strongly suggests that VMP1 promotes apoptosis. Acinar cell death is a hallmark of acute pancreatitis. Necrosis and apoptosis can occur and the mechanisms involved in cell death as well as in tissue recovery were extensively studied (26). VMP1 overexpression during pancreatitis, leading to cell death, could be part of the defense mechanism allowing recovery after the acute attack. In fact, acinar cells are packed with enzymes, which are potentially harmful because their uncontrolled release would result in autodigestion. Therefore, acinar cells submitted to excessive stress may choose the apoptosis pathway to ensure their rapid elimination by macrophages. Otherwise, cells would die by necrosis, with concomitant release of cytolysis products that trigger inflammation, and the inflammatory cells would further increase local stress through cytokine release. This was supported by observations from Kaiser *et al.* (27), who observed a correlation between the intensity of necrosis and the severity of pancreatitis, the severe forms of pancreatitis being accompanied by little apoptosis, whereas apoptotic cells were frequent in mild, edematous pancreatitis. Moreover, induction of apoptosis prior to acute pancreatitis diminished the severity of pancreatitis (23, 28–33).

VMP1 expression may be part of the pathophysiological pathway leading to morphological changes and acinar cell death during the course of acute pancreatitis. The mechanism by which VMP1 promotes vacuole formation and cell death remains to be elucidated. Further studies leading to a better understanding of the function of VMP1 expression by acinar cells would be of potential clinical relevance.

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